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Naturally Occurring Orthopoxviruses: Potential for Recombination with Vaccine Vectors

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Orthopoxviruses are being increasingly used as live recombinant vectors for vaccination against numerous infectious diseases in humans, domestic animals, and wildlife. For risk assessments and surveillance, information about the occurrence, distribution and ecology of orthopoxviruses in western Europe is important but has mainly been based on serological investigations. We have examined kidneys, lungs, spleens, and livers of Norwegian small rodents and common shrews (*Sorex araneus*) for the presence of orthopoxvirus DNA sequences by PCR with primers complementary to the viral thymidine kinase (TK) gene. PCR amplicons were verified as orthopoxvirus specific by hybridization with a vaccinia virus TK-specific probe. A total of 347 animals (1,388 organs) from eight locations in different parts of Norway, collected at different times of the year during 1993 to 1995, were examined. Fifty-two animals (15%) from five locations, up to 1,600 km apart, carried orthopoxvirus DNA in one or more of their organs, most frequently in the lungs. These included 9 of 68 (13%) bank voles (*Clethrionomys glareolus*), 4 of 13 (31%) gray-sided voles (*Clethrionomys rufocanus*), 3 of 11 (27%) northern red-backed voles (*Clethrionomys rutilus*), 16 of 76 (21%) wood mice (*Apodemus sylvaticus*), and 20 of 157 (13%) common shrews. The previous isolation of cowpox virus from two clinical cases of infection (human and feline) at two of the locations investigated suggests that the viruses detected are cowpox and that some of the virus-carrying small mammalian species should be included among the cowpox virus natural reservoir hosts in Scandinavia and western Europe.

Cowpox virus, vaccinia virus, and some close relatives are classified within the genus *Orthopoxvirus* of the *Poxviridae* family. Infection and disease due to cowpox virus have been described in humans and numerous animal species (5) from Europe and western states of the former USSR. Despite its name, cowpox virus infections seem to be rare in cattle (2), while an increasing number of cowpox virus infections in domestic cats and people have been reported during the last 20 years (6, 7, 21, 38, 43). The natural reservoir species for such viruses have not been conclusively identified, although circumstantial evidence and serological data have led to wide acceptance of the suggestion that some rodent species may serve as natural reservoirs for such viruses (3, 12, 26). Isolation of cowpox virus from rodents has, however, been successful only from Turkmenian big gerbils (*Rhombomys opimus*) and yellow susliks (*Citellus fulvus*) (24) and from a Russian root vole (*Microtus oeconomus*) (34).

The species diversity of orthopoxviruses and their host animals in European wildlife have not been determined. Neither has the epidemiology nor ecology of cowpox viruses been elucidated in detail. Recent serological screenings of Norwegian rodents, common shrews (*Sorex araneus*), and red foxes (*Vulpes vulpes*), as well as carnivores from Sweden and Finland, have indicated that orthopoxviruses are widely distributed in these countries (39, 40). In 1994, the first two recognized Norwegian cases of cowpox virus infection were diagnosed in a woman (30) and a domestic cat (41), respectively. These cases emerged within the same part of the country, but without any obvious epidemiological links between them.

Orthopoxviruses are being utilized as live recombinant vaccine vectors for humans, domestic animals, and wildlife (33). Intraspecies recombination and interspecies recombination for such viruses have been proven, and this quality is being exploited for construction of vaccines and expression vectors (28). It is important to attain more profound knowledge about naturally occurring relatives that genetically engineered orthopoxviruses might encounter.

All published screenings for orthopoxviruses in Europe have so far been based on serological methods (3, 12, 19, 25, 31, 34, 39, 40). Such investigations demonstrate the collective number of animals that have been infected at some time during their lifetime. The aim of the present study was to estimate the prevalence of virus-infected individuals within a population at the moment of sampling, by detecting orthopoxvirus-specific DNA in tissues by PCR. We report evidence of orthopoxviruses in small mammalian wildlife species from western Europe, as well as some traits of viral ecology.

MATERIALS AND METHODS

Animals. All animals (Table 1) were trapped (Ugglan special; Grahn AB, Hillerstorp, Sweden), anesthetized with ether, and bled. Most of the common shrews, however, were dead at the time of trap inspection. The animals were examined for ectoparasites and weighed, and, when possible, the sex was determined before the corpses were frozen on dry ice for further processing in the laboratory.

Preparation of tissue for PCR. From each animal (n = 347), samples from spleen (10 mg), liver (25 mg), kidney (25 mg), and lung (25 mg) were excised and stored separately. All samples were mechanically homogenized before the QIAamp tissue kit (QIAGEN GmbH, Düsseldorf, Germany) was used for DNA extraction, bringing the DNA eluate to a final volume of 200 μl in 10 mM Tris-HCl (pH 9.0). The concentrations of DNA in the eluates ranged from 10

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TABLE 1. Species, number, location, and year of trapping of animals examined for orthopoxvirus infection

Species	No. of animals trapped at location[a]:								Total no. of animals
	1	2	3	4	5	6	7	8	
C. rutilus	4+7+0[b]								11
C. rufocanus	4+7+0					0+2+0			13
C. glareolus					0+0+4		0+9+0	30+25+0	68
M. oeconomus	3+1+0								4
A. sylvaticus				0+0+18	0+0+56		0+2+0		76
M. agrestis			0+2+0						2
M. musculus		0+1+0							1
S. araneus	1+15+0	0+12+0	0+8+0	0+0+17	0+0+70			32+2+0	157
L. lemmus						0+15+0			15
Total									347

[a] Location numbers correspond to the locations shown in Fig. 5.
[b] The first, second, and third numbers refer to the numbers of animals trapped in 1993, 1994, and 1995, respectively.

μg/ml for lung samples to 45 μg/ml for liver samples. Five microliters of this eluate, corresponding to 50 to 225 ng of DNA, was used as a template in the PCR.

Oligonucleotide primers. The thymidine kinase (TK)-PCR was designed to detect all known species belonging to the genus *Orthopoxvirus*. The GCG database (GCG software package, version 8.0; University of Wisconsin Genetics Computer Group, Madison) was used to compare the base sequences of the TK genes from different orthopoxviruses. The primers used for amplification of part of the TK gene were selected from the published sequences of the vaccinia virus TK gene (GenBank accession no. J02425) (17), the monkeypox and variola virus TK genes (GenBank accession no. K02025) (13), and the camelpox virus TK gene (GenBank accession no. S51129) (8). The primers were checked for self-complementarity according to the method of Innis et al. (18). The ortho-TK-1 primer (sense) was 5′-AAAAGTACAGAATTAATTAG-3′ (positions 273 to 292; numbering according to that of Hruby et al. [17]) and the ortho-TK-2 primer (antisense) was 5′-TTCAGATAATGGAATAAGAT-3′ (positions 611 to 592); ortho-TK-1 and ortho-TK-2 were used for the 5′ and 3′ primers, respectively, and were expected to give a PCR amplicon of 339 bp (Fig. 1).

Positive controls for the TK-PCR. VV-WR (vaccinia virus strain Western Reserve, VR-119) and CPV-BR (cowpox virus strain Brighton, VR-302), both received from the American Type Culture Collection (ATCC), Rockville, Md., were propagated in Vero cells (ATCC, 81 CCL) and purified as described elsewhere (42). DNA was extracted from the virions by using the QIAamp tissue kit. Pure ectromelia virus DNA (strain Moscow) was a kind gift from M. Buller (St. Louis University Health Sciences Center, St. Louis, Mo.). One picogram and 10 fg of vaccinia virus DNA were routinely used as a template for the positive controls in the screening.

TK-PCR procedure. Strict precautions were taken to avoid false-positive PCR (20), and all tissue extractions, PCR steps, and blottings or hybridizations were done at separate locations. The TK-PCR was performed with a Gene Amp PCR system 9600 (Perkin Elmer Cetus Corp., Norwalk, Conn.). The reaction volume was 50 μl, and the mixture contained PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin), 0.5 μM (each) primer, 0.2 mM (each) deoxynucleoside triphosphates (Promega Corp., Madison, Wis.), and 2.5 U of thermostable polymerase (AmpliTaq; Perkin-Elmer Cetus Corp.). The final reaction mixture, except for the polymerase, was prepared de novo, split into equal aliquots, and frozen at −20°C before use. The reaction tubes were moved from ice to a 95°C heating block. This temperature was held for 5 min before the cycling program started. Five cycles of denaturation at 95°C for 30 s and then annealing at 53°C for 2 min and primer extension at 72°C for 30 s were followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. After the last cycle, the temperature was held at 72°C for 10 more min to ensure full primer extension. Optimization strategies were performed according to guidelines given by Rolfs et al. (36). Optimization was done with 0.5 μg of mouse genomic DNA as background to mimic the maximum content of sample DNA in a PCR tube upon analysis.

Agarose gel electrophoresis. The TK-PCR amplicons were analyzed by electrophoresis in horizontal 0.9% (wt/vol) agarose gels (SeaKem LE; FMC BioProducts, Rockland, Maine) in 1× TAE buffer (0.04 M Tris-acetate, 1.0 mM EDTA) and with 0.001% (wt/vol) ethidium bromide incorporated for DNA staining. Fifteen microliters of PCR products was mixed with 3 μl of a 6× loading buffer (0.25% [wt/vol] bromphenol blue, 40% [wt/vol] sucrose) and loaded in each well. Ten microliters of a 10×-diluted solution of 1-kb DNA ladder (Gibco, BRL, Gaithersburg, Md.) was used as a DNA size marker. Gels were run in 1× TAE buffer at 120 V for 1 h. The PCR products were visualized and photographed on a UV transilluminator (model TM-40; Chromato Vue, San Gabriel, Calif.).

Restriction endonuclease digestion of TK-PCR amplicons. Amplicons from positive controls were digested with the restriction enzymes *Bsp*DI (New England Biolabs, Inc., Beverly, Mass.) and *Eco*RI (Promega). Fragments were separated in a horizontal 2.5% (wt/vol) agarose gel (MetaPhor, FMC BioProducts) in 1× TAE buffer with ethidium bromide incorporated for DNA staining. Twenty microliters of fragments and 4 μl of 6× loading buffer were loaded in each well. The gel was run in 0.5× TAE buffer at 80 V for 1.5 h.

Southern blot analysis to confirm specific TK-PCR amplification. In order to increase the detection level, we chose to combine PCR with Southern blotting for all samples. The DNA in the gels was denatured and blotted on a Hybond-N nylon membrane (Amersham, Buckinghamshire, England) by standard procedures (37). Blotting and hybridization were executed with 45 ng of probe by standard protocols (11). The hybridizations were performed at 65°C, employing a 226-bp labeled probe with a G+C content of 34% (i.e., under very stringent conditions). The probe was made by *Eco*RI restriction endonuclease (Promega) digestion of the TK-PCR amplicon from VV-WR. The two fragments were separated on a 1.3% agarose gel (SeaKEM LE). The larger fragment (226 bp) was cut out of the gel, purified by centrifugation through a glass microfiber filter (Whatman GF/A; Kent, England) at 15,000 × *g* for 10 min, and labeled with [α-^{32}P]dCTP (10 μCi/μl) (Easytides; Dupont Research Products, Boston, Mass.) by using random primer labeling (Rediprime DNA; Amersham). Following hybridization, the membranes were washed and exposed to Cronex 4 medical X-ray film (Dupont, Bad Homburg, Germany) at −70°C for various times for detection of hybridization signals.

Oligonucleotide primers, conditions, and verification of ATIP-PCR. To be able to exclude ectromelia virus as an origin of detected DNA in the small mammals, we used another pair of primers complementary to the A-type inclusion protein (ATIP) gene of VV-WR and CPV-BR. Because of a heterologous sequence within the ATIP-3 primer binding site (32), this primer is not able to bind to the ectromelia virus ATIP gene, and thus amplification does not take place.

Primers complementary to sequences within the ATIP gene of VV-WR (1) and CPV-BR (15) were originally described by Meyer et al. (27). The sizes of the amplicon were expected to be 564 for VV-WR and 566 bp for CPV-BR. ATIP-PCRs were performed with the same apparatus and with the same volume and ingredients in the premix as for the TK-PCR, except that the ATIP primers were used at final concentrations of 0.2 μM (each). CPV-BR DNA served as a positive control. Running conditions were 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. After the last cycle, 72°C was held for 10 min more. All samples were kept at 4°C before analysis. The further processing of the samples was done under conditions identical to those for the TK-PCR samples, except that the probe was made by labeling 45 ng of a

001 TTAACCAGGTCCCTATTGTTACAGATGGAAGGGTCAAACTTAATAAAGGATATTTGTTCG 60
061 ACTTTGTGATTAGTTTGATGCGATTCAAAAAAGAATCCTCTCTAGCTACCACCGCAATAG 120
121 ATCCTGTTAGATACATAGATCCTCGTCGCAATATCGCATTTTCTAACGTGATGGATATAT 180
181 TAAAGTCGAATAAAGTGAACAATAATTAATTCTTTATTGTCATC***ATG***AACGGCGGACATA 240
241 TTCAGTTGATAATCGGCCCCATGTTTTCAGGT**AAAAGTACAGAATTAATTAG**ACGAGTTA 300
301 GACGTTATCAAATAGCTCAATATAAATGCCTGACTATAAAATATTCTAACGATAATAGAT 360
361 ACGGAACGGGACTATGGACGCATGATAAGAATAATTTTGAAGCATTGGAAGCAACTAAAC 420
421 TATGTGATGTCTTGTAATCAATTACAGATTTCTCCGTGATAGGTATCGATGAAGGACAGT 480
481 TCTTTCCAGACATTGTTGAATTCTGTGAGCGTATGGCAAACGAAGGAAAAATAGTTATAG 540
541 TAGCCGCACTCGATGGGACATTTCAACGTAAACCGTTTAATAATATTTTGA**ATCTTATTC** 600
601 **CATTATCTGAA**ATGGTGGTAAAACTAACTGCTGTGTGTATGAAATGCTTTAAGGAGGCTT 660
661 CCTTTTCTAAACGATTGGGTGAGGAAACCGAGATAGAAATAATAGGAGGTAATGATATGT 720
721 ATCAATCGGTGTGTAGAAAGTGTTACATCGACTCATAATATTGTATTTTTTATCTAGAAC 780
781 TAAATAACATTGAT

FIG. 1. Nucleotide sequence of the vaccinia virus TK gene and its flanking regions (17). The positions of the PCR primers are shown in boldface and are underlined. The *Bsp*DI (position 465) and *Eco*RI (position 498) cleavage sites are underlined. The start codon for translation is shown in boldface and italics.

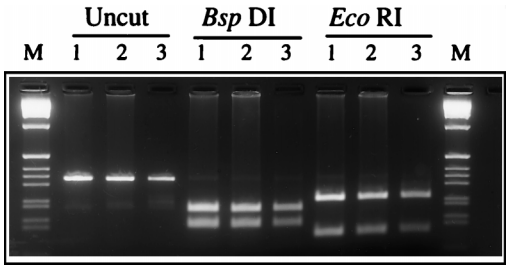

FIG. 2. Agarose gel showing TK-PCR amplicons of VV-WR, CPV-BR, and ectromelia virus (strain Moscow) and restriction fragments from *Bsp*DI and *Eco*RI digestion of the amplicons. Lanes: 1, VV-WR; 2, CPV-BR; 3, ectromelia virus (strain Moscow); M, 1-kb size marker.

165-bp internal *Bgl*II (New England Biolabs, Inc.) fragment of the CPV-BR ATIP-PCR amplicon.

RESULTS

Specificity and sensitivity of the PCR method. According to computer-based sequence analysis, the TK primers could promote the amplification of DNA from all orthopoxvirus species that have been demonstrated in Europe. A 100% identity in a 20-bp overlap was found for both primers when the GCG database TK sequences for vaccinia virus, monkeypox virus, and variola virus were aligned. For camelpox virus, there was one mismatch at the 3′ end of the ortho-TK-2 primer. The TK-PCR amplicons from VV-WR, CPV-BR, and ectromelia virus DNA appeared as a single DNA band with a size close to that of the 344-bp band of the 1-kb ladder.

The identities of the PCR amplicons as orthopoxvirus TK gene products were verified by two procedures. The PCR amplicons from the orthopoxvirus reference strains were (i) digested with the *Eco*RI (Promega) and *Bsp*DI (New England Biolabs, Inc.) endonucleases and shown to yield the expected fragment patterns (Fig. 2) and (ii) hybridized with a [α-^{32}P] dCTP-labeled probe representing 226 bp of the vaccinia virus TK gene (Fig. 3). No hybridization could be detected for fowlpox virus DNA (ATCC, VR-229).

The lowest level of detection for the TK-PCR was found by testing 10-fold dilutions of vaccinia virus DNA in a constant background of mouse genomic DNA (0.5 μg). Amplification of

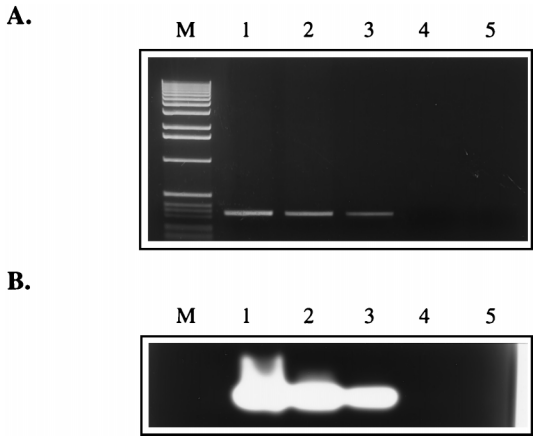

FIG. 3. (A) Agarose gel showing TK-PCR amplicons of VV-WR, CPV-BR, and ectromelia virus (strain Moscow). (B) Southern hybridization blots of the agarose gel in panel A when hybridized with the 226-bp vaccinia virus TK-specific labeled probe. Lanes: 1, VV-WR; 2, CPV-BR; 3, ectromelia virus (strain Moscow); 4, fowlpox virus; 5, negative control (H_2O); M, 1-kb marker.

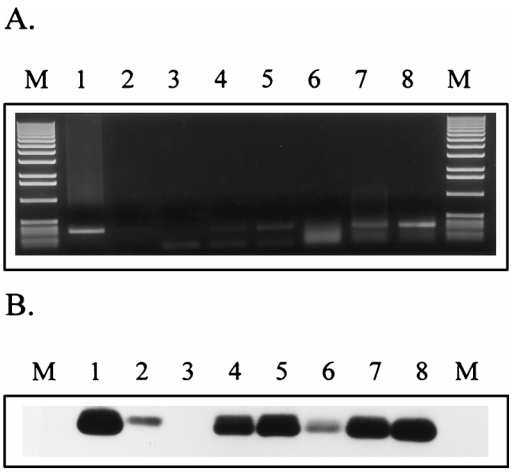

FIG. 4. (A) Agarose gel showing examples of TK-PCR amplicons from small mammal tissue samples. (B) Southern hybridization blots of the same samples from panel A. Lanes: 1 and 2, VV-WR-positive control, with 10 and 1 μl of amplicon, respectively; 3, negative control (H_2O); 4 and 5, lung DNA from two *Sorex araneus* animals; 6, 7, and 8, DNA from liver, spleen, and kidney, respectively, from three *Clethrionomys glareolus* animals; M, 1-kb size marker.

mouse genomic DNA alone did not result in any visible PCR products or signals after hybridization. As little as 1 pg of vaccinia virus DNA could be detected, and according to a vaccinia virus genomic weight of 2.6×10^{-10} μg given by Holowczak (16), 1 pg of DNA corresponds to approximately 4,000 viral genomes. When PCR was combined with blotting or hybridization, the detection level increased 100-fold (i.e., 10 fg of vaccinia virus DNA corresponding to 40 viral particles could be detected [data not shown]).

The combination of PCR with blotting or hybridization revealed a higher number of positive results than with PCR alone (Fig. 4). Of a total number of 1,388 samples, only 14 samples turned out positive by PCR alone, whereas after blotting and hybridization, the number of positive samples increased to 59 (i.e., the PCR alone could detect only 24% of the true-positive samples). DNA samples from the 59 PCR- or hybridization-positive organs were analyzed a second time in the same assay.

The ATIP-PCR was tested with DNA from VV-WR, CPV-BR, ectromelia virus (strain Moscow), and FPV, and only the two former DNAs were amplified, suggesting that the detected DNA did not have its origin in ectromelia virus. Amplicons were verified by *Bgl*II digestion (data not shown). The sensitivity of the ATIP-PCR was not determined.

Occurrence and distribution of orthopoxviruses in Norway. A total number of 1,388 organs representing 347 animals were examined for the presence of orthopoxvirus TK gene sequences. The number and origin of animals collected are shown in Table 1. Orthopoxvirus-specific DNA was detected in five of nine different animal species from five of eight locations (Fig. 5, locations 1, 4, 5, 6, and 8) and in 59 organs from 52 individual animals (Table 2). Virus DNA could be detected in wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*), northern red-backed voles (*Clethrionomys rutilus*), gray-sided voles (*Clethrionomys rufocanus*), and common shrews. No statistically significant correlation was found between prevalence and species. Thus, no specific species could be pointed out as a one-reservoir host. Concerning different tissues, 13.5% of the lung samples were positive, compared to 0.8, 1.4, and 2.0% of the liver, kidney, and spleen samples, respectively.

No orthopoxvirus DNA was detected in field voles (*Microtus*

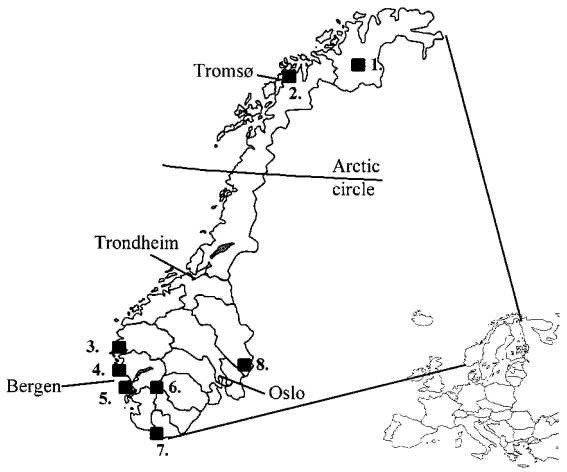

FIG. 5. Trapping locations of wild rodents and common shrews. Local names and counties are given. 1, Masi, Finnmark; 2, Tromsø, Troms; 3, Bygstad, Sogn og Fjordane; 4, Austrheim, Hordaland; 5, Kalandsvatn, Hordaland; 6, Hardangervidda National Park; 7, Søgne, Vest-Agder; 8, Kongsvinger, Hedmark.

agrestis), root voles, house mice (*Mus musculus*), or Norway lemmings (*Lemmus lemmus*), but it should be emphasized that for some of the trapping locations and for some of the animal species, the collections were far too small to be representative (Table 1).

While 13 of 74 (18%) small mammals collected in 1993 and 38 of 165 (23%) of those collected in 1995 were orthopoxvirus infected, none of the 108 animals trapped in 1994 was carrying virus, except for 1 gray-sided vole (0.9%) collected at Hardangervidda in August. We have no obvious explanation for this discrepancy.

Forty-one of the 59 TK-PCR-positive organ samples were also positive by ATIP-PCR or hybridization and are included in Table 2.

No clinical signs of disease were recorded for the animals. An attempt to isolate virus from lung, liver, spleen, and kidney from four bank voles, six wood mice, and one common shrew, all positive with regard to orthopoxvirus DNA (TK-PCR), failed (39).

DISCUSSION

There are at least three good reasons to investigate the occurrence, distribution, and characteristics of zoonotic orthopoxviruses. First, one viral species, cowpox virus, is a proven pathogen for humans and some domestic animals (5, 29). Second, the potential of such viruses to evoke disease in wildlife species is unknown, although it is well documented that they are highly pathogenic for several zoo-kept animals (4, 23, 34, 44). Third, the use of vaccinia virus and other orthopoxviruses as live vaccine vectors (33) makes the prospect of recombinant progeny between naturally circulating and released or escaped genetically modified orthopoxviruses an urgent matter.

The geographical distribution of the various small rodent species had a major impact on the selection of trapping locations. In general, field voles, Norway lemmings, and common shrews are found all over the country. The bank vole is present in most parts of Norway, except the northernmost part, where the northern red-backed vole lives. Gray-sided voles are found all over the country, except along the coast in the southernmost part. The wood mouse lives along the coast in the southern part of Norway. The root vole is present in mountain areas in the south and cold areas in the north. In order to cover such a spectrum of ecosystems, both inland and coastal ecosystems representative of the country were selected. We included new trapping locations in 1995, taking into consideration the two clinical cases of cowpox virus infection that appeared the previous year (Fig. 5, locations 4 and 5).

Information about the distribution of orthopoxviruses within the bodies of small mammalian species that are present in Norway was lacking. We chose target tissues for DNA extraction and TK-PCR analysis based on information about ectromelia virus tissue tropism in laboratory mice (9) and successful attempts at isolation of cowpox virus from rodents (25), although these involved rodent species not present in Norway.

In the present study, we used a primer set complementary to the highly conserved orthopoxvirus TK gene to be able to detect several members of the orthopoxvirus genus (8). Of a total of 1,388 organs, 59 organ samples contained orthopoxvirus DNA (Table 2). The ATIP-PCR detected 69% of the TK-PCR-positive samples. The dropping out of 31% of the samples is probably due to an ATIP-PCR with a lower sensitivity than the TK-PCR. Comparison of the two PCR assays for animals that

TABLE 2. Distribution of individuals with orthopoxvirus DNA at the geographical locations and time of trapping where positive individuals are represented

Location[a] (yr/mo)	Species	No. of animals			PCR-positive organ(s)[c] (no. of samples)	
		Positive/total (%)	Male/female	Adult/subadult[b]	TK-PCR	ATIP-PCR
1 (1993/6)	*C. rufocanus*	3/4 (75)	3/0	3/0	Lu (3)	Lu (3)
	C. rutilus	3/4 (75)	0/3	3/0	Lu (3)	Lu (3)
4 (1995/8, 9)	*A. sylvaticus*	3/18 (17)	3/0	3/0	Lu (3)	Lu
	S. araneus	1/7 (14)	1/0	1/0	Lu	Lu
5 (1995/8, 9)	*A. sylvaticus*	13/56 (23)	6/7	5/8	Lu (12), Li, K (2), S (4)	Lu (9), Li, K, S
	C. glareolus	2/4 (50)	1/1	1/1	Lu (2)	Lu
	S. araneus	19/70 (27)	6/13	18/1	Lu (19)	Lu (13)
6 (1994/8)	*C. rufocanus*	1/2 (50)	1/0	1/0	Lu	Lu
8 (1993/8)	*C. glareolus*	7/30 (23)	6/1	3/4	Lu (3), Li, K (2), S (2)	Lu (2), Li, K, S (2)

[a] Location numbers correspond to the locations shown in Fig. 5.
[b] Based on body weight.
[c] Lu, lung; Li, liver; K, kidney; S, spleen.

were TK positive for two or more organs indicates that this is correct.

Several of the known orthopoxvirus species have been isolated from rodents (10, 24, 35), and ectromelia virus, formerly believed to infect laboratory mice only (14), has recently been isolated from fur-bearing foxes and minks in the Czech Republic (22). Since the ATIP-PCR used in this study is unable to detect DNA from ectromelia virus but revealed positive samples among the mammals investigated, we conclude that the origin of the orthopoxvirus DNA is not ectromelia virus. Furthermore, we think that it is unlikely that vaccinia virus is endemic in the small mammals examined here. It is more reasonable to believe that some cowpox virus or viruses are involved. Final conclusions concerning the identity of Norwegian orthopoxviruses circulating in small mammal populations can only be done by isolation and characterization.

Common shrews represent 45% of our total animal collection and were trapped at all locations, except Søgne and Hardangervidda. TK-PCR-positive common shrews were only found at Austrheim and Kalandsvatn (Fig. 5, locations 4 and 5). The fact that rodent species but no common shrews were TK-PCR positive at other locations may reflect qualitative differences in the distribution of virus in small mammal colonies at different sites. On the other hand, the explanation may be that none of the species included in these investigations are real reservoir animals, but are running through infections newly received from some as-yet-unidentified reservoir species. This would again imply that the amount of contact between these unknown natural reservoirs and common shrews differs among our trapping locations.

We still do not know whether any of the orthopoxvirus-infected species are natural reservoirs for such viruses, but the body weight and the time of collection for some virus-infected individuals provide leads for further work. For instance, six virus-infected gray-sided voles and northern red-backed voles collected in June 1993 in Masi (Fig. 5, location 1) had body weights ranging from 29 to 50 g. These animals must have overwintered, and if they have been persistently infected from the preceding year, these vole species should be considered a natural orthopoxvirus reservoir. Another situation is illustrated by the bank vole collection from Kongsvinger in August 1993 (Fig. 5, location 8). The animals with body weights ranging from 13.5 to 18 g have most certainly been born that summer season, and one may therefore assume that orthopoxvirus activity and transmission have occurred that season.

Previous serological surveys of British wildlife have detected orthopoxvirus-specific antibodies in several rodent species, including bank voles and wood mice (12, 19), but unfortunately, no final conclusions concerning the species of orthopoxvirus eliciting these antibodies could be drawn. The same investigations also demonstrated extreme variations in prevalence between different geographical locations, similar to those recorded in the serological studies in Norway as well as in the present study. Serological assays are unable to detect acute infections in which seroconversion has not occurred. Antibody prevalence may give an estimate of the total infection rate, but does not reveal the frequency of virus carriers at any given time. PCR gives the prevalence of virus-infected individuals within a population at the moment of sampling and important information about the organs involved in an infection. However, by using PCR as a screening method for infections with an unknown pathogenesis, there is a risk of missing organs involved in infection.

Lung samples are heavily overrepresented among the positive samples. This may indicate that the respiratory organs are ports of entry into the organism, but may also be a phenomenon secondary to systemic spread of virus. Orthopoxvirus was, to a lesser extent, detected in spleens, livers, and kidneys, allowing the conclusion that orthopoxvirus or viruses may cause systemic infections in small wild mammals. It is, however, still unknown whether disease is ever evoked. In Turkmenia, cowpox viruses were isolated from big gerbils and yellow susliks that were apparently healthy (25), and we have not observed any clinical symptoms among the animals collected so far.

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